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TOROVIRUSES *(CORONAVIRIDAE)*

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History

Toroviruses were defined in the early 1980s as a result of a trilateral, partly collaborative study between groups in Berne, Switzerland (isolation in cell culture), Ames/Iowa, USA (discovery of Breda virus and propagation in calves) and Utrecht, The Netherlands (biologic and molecular characterization).

An equine torovirus (ETV), originally referred to as Berne virus, was accidentally isolated in equine kidney cells in 1972 from a rectal swab taken from a horse with diarrhea. Upon post-mortem examination pseudomembranous enteritis and miliary granulomas and necrosis in the liver were diagnosed; Salmonella Lille (O, 6, 7, Z_{38}) was considered to be the causative agent. ETV can be propagated in lines of equine dermis or embryonic mule skin cells, where it causes a cytopathic effect that results in cell lysis. The virus was not neutralized by antisera against known equine viruses. Serologic crossreactions were observed in neutralization and ELISA with sera from calves that had been experimentally infected with morphologically similar particles, the Breda viruses.

A bovine torovirus (BTV), first described as Breda virus was discovered in 1979 during investigations in a dairy herd in Breda (Iowa), in which severe neonatal calf diarrhea had been a problem for three consecutive years. Despite repeated attempts, BTV has not been adapted to growth in cell or tissue culture, which has hampered its biochemical, biophysical and molecular characterization. The pathogenesis and pathology of BTV infections have been studied in gnotobiotic calves.

Torovirus-like particles have been seen in EM preparations from fecal samples of pigs and humans (children and adults); proof that the observed structures are not artefacts was obtained when toroviral RNA sequences were found in the feces of piglets and in stools from humans with diarrhea.

Taxonomy and Classification

The name of the viruses assembled in this genus is derived from the term *torus* (Latin) which designates the lowest convex molding in the base of a column or pilaster. It refers to the biconcave disk or doughnut shape of the virion that is determined by a tubular capsid of helicoidal symmetry, which is surrounded by a peplomer-bearing envelope. In addition to the unique toroid form, virions may also appear as straight or bent rod-shaped particles.

Analysis of the genetic information and replication mode of the prototype ETV showed that toroviruses are evolutionarily related to the *Coronaviridae* and, to a lesser extent, to the *Arteriviridae*. However, the lack of sequence homology in the structural genes and the absence of antigenic relatedness with coronaviruses justify their separate taxonomic position as a genus, *Torovirus*, in the *Coronaviridae* family.

The families *Coronaviridae* and *Arteriviridae* form the Order *Nidovirales* (from *nidus*, Latin for 'the nest' – alluding to the nested set of subgenomic RNAs transcribed during replication), the second order recognized in animal virus taxonomy so far.

Virion Properties

Extracellular ETV particles possess a helical nucleocapsid coiled into a hollow tube (diameter 23 nm, average length 104 nm, periodicity 4.5 nm) which is either straight or bent into an open torus. A tightly fitting envelope, 11 nm thick, surrounds this structure. Consequently, the virion may assume an erythrocyte-like or a kidney shape, depending on whether the envelope follows the small curvature of the nucleocapsid or not. The largest diameter of ETV is estimated at 120–140 nm. Club-shaped projections (average length 20 nm) are present on the particle surface.

In the cell, predominantly rod-shaped particles are encountered. Cross-sections through tubular virions appear as three concentric circles of high electron density with an electron-lucent center.

Negatively stained BTV virions appear to be either kidney- or C-shaped, measuring 30–120 nm, or approximately circular, measuring 75–90 nm in diameter. Their envelope bears prominent drumstick-shaped peplomers (17–24 nm), and a fringe of shorter spikes 8–10 nm in length. The short projections represent the hemagglutinin esterase protein, which is present on the surface of bovine torovirions. In intestinal cells of calves killed 48–96 h after infection, tubules of 21 nm diameter and indeterminate length were found both in the cytoplasm and in nuclei.

In thin-sectioned preparations, intracellular torovirions show a bacilliform morphology (rods with both ends rounded – in contrast to the circular outline of coronavirions); extracellular particles may reveal twin circular structures resulting from cross-sections through both limbs of the C-shaped tubular nucleocapsid.

A model of a torovirion is given in **Fig. 1**.

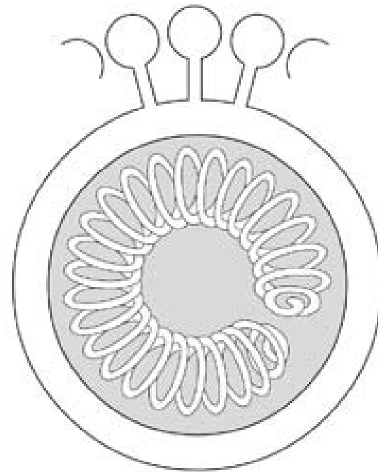

Figure 1 A schematic model of a torovirion. Illustration by Ank Klein-Willink.

Genome

ETV virions (sedimentation coefficient 380S) contain one species of polyadenylated RNA of positive polarity; in agarose gel electrophoresis its length appears as ≥20 kb. When assayed under hypertonic transfection conditions genomic RNA is found infectious and RNase-sensitive.

Proteins

Proteins with molecular weights of 20 kDa, 22 kDa, 37 kDa, and 80–100 kDa are identified in labeled ETV virions. Detergent treatment releases the 22, 37 and 80–100 kDa species from the virion, which indicates their association with the envelope. Only the 20 kDa protein is present in purified ETV nucleocapsids and was accordingly named nucleocapsid (N) protein. The heterogeneous, *N*-glycosylated 80–100 kDa protein is recognized by both neutralizing and hemagglutination-inhibiting monoclonal antibodies and is therefore identified as the peplomer (P) protein. Another membrane-associated polypeptide is the non-glycosylated envelope protein (E; 22 kDa); the 37 kDa molecule also occurs in close association with the viral membrane, but its virus specificity could not be established.

From the deduced amino acid sequence of the nucleocapsid (N) protein gene a basic protein of 18.3 kDa is predicted. *In vitro* transcription and translation, followed by immunoprecipitation, were used to identify the gene. Identification was confirmed by metabolic labeling, using the knowledge that cysteine residues are absent from the amino acid sequence of the N protein. Smaller N-related polypeptides encountered in ETV-infected cell lysates are products of aberrant translation, due to initiation on AUG codons further downstream in the N protein gene.

The 26.5 kDa product of the ETV membrane (M) protein gene was identified by *in vitro* transcription and translation. Computer analysis revealed the characteristics of a class III membrane protein lacking a cleaved signal sequence but containing three successive transmembrane α-helices in the N-terminal half. Only small portions of either end of the polypeptide are exposed on opposite sides of the vesicle membranes; the C-terminus protrudes at the cytoplasmic side of the membrane. The M protein accumulates in intracellular membranes, predominantly those of the endoplasmic reticulum.

The nucleotide sequence of the peplomer or spike (S) protein gene encodes an apoprotein of 1581 amino acids with an M_r of about 178 kDa. The deduced amino acid sequence contains domains typical for type I membrane glycoproteins: an N-terminal signal sequence, a putative C-terminal transmembrane anchor, and a cytoplasmic tail.

Since BTV has not been labeled in cultured cells, its protein composition was studied by means of surface radioiodination of purified virus. Polypeptide species of 105, 85, 37, and 20 kDa were identified, of which the former two probably represent the BTV peplomeric surface structures. Rabbit antisera raised against purified BTV recognize the ETV S protein in immunoprecipitation experiments.

Physical Properties

Buoyant densities of 1.16, 1.18 and 1.14 g ml^{-1} are reported for ETV, BTV serotype 2 and human toroviruses, respectively.

Under experimental conditions ETV is remarkably stable, even to the action of phospholipase C or deoxycholate; Triton X-100 and organic solvents destroy its infectivity. BTV1 appears to be less stable than ETV, as changes in its sedimentation behavior and density have been observed after prolonged storage at −70°C. The infectivity of a fecal preparation containing BTV1 was lost completely after 3 weeks at 4°C whereas ETV in cell-free supernatant remained stable for 92 days. Two cycles of freeze–thawing of purified BTV2 resulted in loss of peplomers.

Replication

Equine torovirus (strain Berne) replication occurs in the cytoplasm via a 3′-coterminal nested set of five subgenomic mRNAs. Preformed tubular capsids bud through membranes of the Golgi stack and of the endoplasmic reticulum. A host cell nuclear function seems to be required since UV preirradiation of cells, actinomycin D and α-amanitin reduce virus yields.

Transcription

In ETV-infected cells five virus-specific polyadenylated RNA species are found with >20.0, 7.5, 2.1, 1.4, 0.8 kb.

Northern (RNA) blot hybridizations with restriction fragments from cDNA clones showed that the five ETV mRNAs form a 3′-coterminal nested set. Sequence analysis revealed the presence of four complete open reading frames of 4743, 699, 426 and 480 nucleotides, with initiation codons coinciding with the 5′ ends of ETV RNAs 2 through 5, respectively; RNA 5 is contiguous on the consensus sequence. ETV RNAs 1, 2 and 3 are transcribed independently, as shown by UV transcription mapping. Upstream of the AUG codon of each open reading frame a conserved sequence pattern is encountered, probably a core promoter sequence in subgenomic RNA transcription. In the area surrounding the core promoter region of the two most abundant subgenomic ETV RNAs, a number of homologous sequence motifs occur.

Translation

The 7.5, 2.1 and 0.8 kb RNAs encode a 151 kDa product (possibly the precursor to the S protein), the M protein, and the N protein, respectively, as shown by *in vitro* translation.

The 3′ part (8 kb) of the polymerase gene of ETV contains at least two open reading frames (designated ORF 1a and ORF 1b) which overlap by 12 nucleotides. The complete sequence of ORF 1b (6873 nucleotides) is known. Like corona- and arteriviruses, ETV expresses its ORF 1b by ribosomal frameshifting during translation of the genomic RNA; also the predicted tertiary RNA structure (a pseudoknot) in the frameshift-directing region is similar. The amino acid sequence of the predicted ETV ORF 1b translation product contains homologies with the ORF 1b product of coronaviruses. Four conserved domains are present: the putative polymerase domain, an area containing conserved cystein and histidine residues, a putative helicase motif, and a domain apparently unique for toro- and coronaviruses.

Post-translational Processing

The *N*-glycosylated peplomer protein is derived from processing of a 200 kDa precursor present in infected cells but not in virions. Eighteen potential *N*-glycosylation sites, two heptad repeat domains, and a possible 'trypsin-like' cleavage site exist in the peplomer gene. The mature S protein consists of two subunits and their electrophoretic mobility on endoglycosidase F treatment suggests that the predicted cleavage site is functional *in vivo*. The heptad repeat domains are probably involved in the generation of an intra-chain coiled-coil secondary structure; similar interchain interactions can play a role in the formation of the observed S protein dimers. The intra- and interchain coiled-coil interactions may stabilize the elongated ETV peplomers.

Assembly

About 10 h after infection ETV particles are seen within parts of the unaltered Golgi apparatus and extracellularly. At that time, tubular structures of variable length, diameter and electron-density appear in the cytoplasm and in the nucleus of infected cells, probably representing preformed nucleocapsids. It is unknown whether the accumulation of nucleocapsids in the nucleus reflects a nuclear phase in the replication of ETV or some sort of defective assembly.

Viruses predominantly bud into the lumen of Golgi cisternae. The preformed nucleocapsid tubules approach the Golgi membrane with one of both rounded ends and attach to it along one side. During budding the nucleocapsid is apparently stabilized, leading to a higher electron density and a constant diameter (23 nm).

Intracellular BTV virions are rod-shaped with rounded ends; they measure 35–40 nm in diameter and are 80–100 nm long.

Defective Interfering Virus

Defective interfering (DI) genomes of ETV can be generated by serial undiluted passages. Isokinetic sucrose gradient analysis showed that they are packaged into virus-like particles with lower S values than standard virions. DI RNAs contain sequences from the presumed 5′ end and the proven 3′ end of the ETV genome. Using probes from the 5′ end, a consensus nucleotide sequence of about 800 nt and the 5′ end of the putative ETV polymerase gene were identified. A conserved sequence motif, probably involved in subgenomic RNA transcription, is situated immediately downstream of the 5′ end of the DI RNAs. There is no evidence for the presence of a common leader sequence in ETV RNAs.

In the gut of a BTV-infected calf, a wave of simultaneous infections progresses through a population of susceptible cells. In view of the immense particle numbers encountered in the feces, enteroabsorptive epithelial cells are probably infected at high multiplicities; DI particles may also be generated *in vivo* and may play a role in modulating the pathogenesis of torovirus infections.

Geographic and Seasonal Distribution

Toroviruses in cattle have been evidenced by ELISA serology in Europe, North America and Asia. Seasonal patterns of infection have been described in calves in relation to herd management (pasture/stable). Most adult horses in Switzerland possess neutralizing antibodies to ETV. Possible human toroviruses have been found in France, Great Britain, The Netherlands and the USA.

Host Range and Virus Propagation

By using the neutralization assay, antibodies to ETV were found in sera from horses, cattle, goats, sheep, pigs, rabbits and feral mice, but not in humans or in carnivores. Torovirus-like particles have been seen in fecal samples of cats with a transmissible diarrhea, but neither serologic nor molecular identification was obtained.

By electron microscopy, pleomorphic virions have been observed in the feces of children and adults with diarrhea; the particles were coated and aggregated after the addition of anti-BTV calf sera. The stool specimen reacted in an ELISA for the detection of BTV antigen in calves, and possessed a low titer of hemagglutinin for rat erythrocytes, which was blocked by antisera to BTV.

Toroviral RNA sequences have been evidenced in the stools from humans with diarrhea. The use of fresh material (avoiding freeze/thawing) is essential for obtaining unequivocal results. Sequence analysis of RNA extracted from specimens of pediatric patients and amplified by reverse transcriptase–polymerase chain reaction (RT-PCR) showed a high degree of identity with the corresponding ETV sequence. This is surprising in view of the observation that the divergence between porcine and bovine/equine torovirus sequences is greater than between human and bovine/equine torovirus sequences. If confirmed, this would indicate occasional spillover of the infection from ungulates to humans, rather than human-to-human transmission.

Cultured cells of equid origin (horse, mule) can be infected with ETV; no other cell species tested supports viral growth. Bovine toroviruses could not be propagated in any culture of primary cells or permanent lines and had to be passaged in gnotobiotic calves. Putative torovirus isolations from young calves with respiratory symptoms (pneumonia) in MDBK cells have been challenged, and a bovine coronavirus was identified in the culture.

Genetics

No information is available.

Evolution

Toroviruses and coronaviruses are ancestrally related by divergence of their polymerase and envelope proteins from common ancestors. In addition, their genome organization and expression strategy, which involves the synthesis of a 3′-coterminal nested set of mRNAs, are comparable. Four domains of amino acid sequence homology exist in the product of ORF 1b of the *POL* gene, which underlines the existence of an evolutionary relationship. In view of these findings, toro- and coronaviruses have been classified as separate genera in the family *Coronaviridae*, which, together with the *Arteriviridae* forms the order *Nidovirales*.

Nucleotide sequence analysis of the ETV genome has revealed the results of two independent nonhomologous RNA recombinations: ORF 4 encodes a protein with significant sequence similarity (30–35% identical residues) to a part of the hemagglutinin esterase proteins of coronaviruses and of influenza virus C. Although this gene is truncated in ETV, it is intact and translated into a functional, enzymatically active protein in BTV; this product is visible as a

second fringe of (short) projections on the viral envelope.

The sequence of the C-terminal part of the predicted ETV polymerase ORF 1a product contains 31–36% identical amino acids when compared with the sequence of a nonstructural 30/32 kDa coronavirus protein. The cluster of coronaviruses which contains this nonstructural gene does not express it as a part of their polymerase, but by synthesizing an additional subgenomic mRNA.

Serologic Relationships and Variability

One strain of ETV has been isolated (and re-isolated from the same material), but all attempts to obtain a second equine isolate were fruitless.

Two strains of BTV have been reported in addition to the original isolate by Woode and colleagues: one had been detected in feces from a 5-month-old diarrheal calf in Ohio, and a second Iowa strain was recovered from a 2-day-old experimental animal. On the basis of their reactivity in ELISA, immune electron microscopy and hemagglutination/hemagglutination inhibition assays using rat erythrocytes the three isolates were assigned to two serotypes: BTV1, represented by the Iowa 1 isolate, and BTV2 comprising the Ohio and the second Iowa isolate.

The occurrence of antigenically different toroviruses is not unlikely. Two serotypes of BTV have been described and more probably exist. It is anticipated that serologically unrelated toroviruses will be identified with the aid of nucleic acid probes.

Epidemiology

The high prevalence of BTV antibodies in cows cannot be explained by the few infections found in calves and cows with diarrhea. The viruses may circulate in herds through inapparently or chronically infected animals, as described for rota- and coronaviruses. The level of maternal BTV-specific antibodies influences the clinical outcome of the infection, as differences in the severity of diarrhea were observed between colostrum-fed and colostrum-deprived animals.

With the aid of solid-phase immune-electron microscopy, torovirions can be identified in fecal material; without this selection, virion pleomorphism makes diagnosis by electron microscopy ambiguous.

Transmission and Tissue Tropism

Toroviruses probably spread through feco-oral contact. In feces samples from experimentally BTV-infected calves HA titers in excess of 10^7 units ml^{-1} are measured which would correspond to particle concentrations of $>10^{11}$. Therefore, once an outbreak is under way the infection spreads rapidly, especially when highly susceptible animals are on the premises (e.g. in the calving season).

Using RT-PCR, torovirus sequences were obtained from fecal samples of weaning piglets; an association with weaning diarrhea has not been established.

Pathogenicity

Torovirus infections play a role in diarrhea of breeding calves up to two months of age, and in winter dysentery of adult cattle. Torovirus was detected four times more often in diarrheal calves than in healthy animals. Torovirus-associated diarrhea of calves started later (average 12.7 days of age) than enteritis due to rota- or coronaviruses (average 7.7 and 8.3 days, respectively). Seroconversion was found significantly more often after winter dysentery outbreaks than on farms without a disease history; coronavirus seroconversion was less common.

Clinical Features of Infection

Seroconversions to ETV occurred in all horses between 10 and 12 months of age, but without symptoms. Experimentally infected animals (intravenous route) seroconverted without clinical signs. To the author's knowledge oral infection experiments in horses have not been reported so far.

All BTV strains are pathogenic – although with varying virulence – for newborn gnotobiotic and nonimmune conventional calves after oral infection. Most calves develop anorexia, a watery, yellow–green diarrhea that lasts 4–6 days, and shed virus for 3–4 days. In some calves the diarrhea is preceded by a mild temperature reaction (40°C). In the calves with a normal intestinal flora the diarrhea is generally more severe than in gnotobiotic calves. Reduction of D-xylose resorption may reach 65% in severely affected calves. In some animals depression and dehydration is observed, occasionally with shivering, hyperpnea and watery eye discharge. Mortality in experimental infections approaches 25%.

Pathology and Histopathology

Target organs of BTV in calves are the lower half or two-thirds of the small intestine and the entire large intestine, particularly the spiral colon. There is little macroscopic evidence of the infection. Histological examination shows villous atrophy and epithelial desquamation from the mid-jejunum to the lower small intestine, and areas of necrosis in the large intestine. Both crypt and villus epithelial cells contain antigen as shown by immunofluorescence. The

watery diarrhea is probably a result of loss of resorptive capacity of the colonic mucosa, combined with malabsorption in the small intestine. Infection of crypt epithelium may affect the duration of diarrhea, as regeneration of villus epithelium starts in the crypts. The germinal centers of the Peyer's patches are depleted of lymphocytes and may occasionally show fresh hemorrhage. The dome epithelial cells, including the M cells, display the same cytopathic changes as seen in the absorptive cells of villi. Virions are found in cells of both the small and large intestine. Extracellular virus is closely associated with microvilli of absorptive cells and in the coated pits between microvilli, indicating receptor-mediated endocytosis. In addition, virions are found between enterocytes at the basal and lateral plasma membranes. Virions in various stages of degradation are found within macrophages in the lamina propria.

Antigen is detected as early as 48 h after infection in epithelial cells of the lower half of the villus and of the crypts of the affected areas, as well as in dome epithelium. Fluorescence is cytoplasmic (although a few nuclei may be faintly stained) and generally most pronounced in the intestines with the least tissue damage. The mid-jejunum is infected first, the infection eventually reaching the large intestine. Diagnosis by immunofluorescence test (IFT) should be performed preferentially on sections of the large intestine from calves killed after the onset of diarrhea (i.e. several days after the infection of epithelium).

Immune Response

Up to the age of 4 months, all calves in a sentinel experiment regularly excreted BTV in the feces. They showed early serum IgM responses despite the presence of IgG1 isotype maternal antibodies, but no IgA seroconversion. Antibody titers then decreased below detection, persistent IgG1 titers developed in only a few animals. After introduction into the dairy herd at 10 months of age, all calves developed diarrhea and shed virus. Seroconversion for all antibody isotypes was observed at this stage, indicating lack of mucosal memory. In contrast, coronavirus infection in the presence of maternal antibodies leads to isotype switch and a memory response.

Prevention and Control

No control strategies have been implemented.

Future

The pathogenic significance of toroviruses for animals and humans, also as agents of nonenteric infections needs to be established. Diagnostic procedures for the discovery of more distantly related viruses of this cluster will have to include procedures for the recognition of nucleotide sequence motives.

See also: **Arteriviruses (*Arteriviridae*); Defective interfering viruses.**

Further Reading

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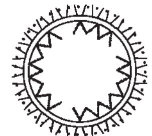
TOSPOVIRUSES (*BUNYAVIRIDAE*)



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History

Diseases incited by tomato spotted wilt virus (TSWV) were first reported in 1915 and were considered to be of viral etiology by 1930. This taxon of plant viruses was categorized as a monotypic virus group consisting of a single virus (tomato spotted wilt virus; TSWV) until the report of Impatiens necrotic spot